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Review

Brain gangliosides in axon–myelin stability and axon regeneration

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ABSTRACT

Gangliosides, sialic acid-bearing glycosphingolipids, are expressed at high abundance and complexity in the brain. Altered ganglioside expression results in neural disorders, including seizures and axon degeneration. Brain gangliosides function, in part, by interacting with a ganglioside-binding lectin, myelin-associated glycoprotein (MAG). MAG, on the innermost wrap of the myelin sheath, binds to gangliosides GD1a and GT1b on axons. MAG–ganglioside binding ensures optimal axon–myelin cell–cell interactions, enhances long-term axon–myelin stability and inhibits axon outgrowth after injury. Knowledge of the molecular interactions of brain gangliosides may improve understanding of axon–myelin stability and provide opportunities to enhance recovery after nerve injury.

1. Introduction

Gangliosides, sialic acid-bearing glycosphingolipids expressed on all vertebrate tissues, are found in relatively high abundance and structural complexity in the brain, where they represent ~1% of total brain lipids [1,2]. The ceramide lipid moieties of gangliosides are embedded in the outer leaflet of the plasma membrane, and their complex glycans extend into the extracellular space [3]. As major cell surface structural determinants, gangliosides are positioned to interact laterally with other molecules in their own membranes and with molecules on apposing cell membranes [4,5]. Lateral (*cis*) molecular interactions in the plane of the membrane result in ganglioside-mediated regulation of membrane proteins (such as receptor kinases) whereas interactions with proteins on apposing membranes (*trans*) result in ganglioside-mediated cell–cell recognition. When *trans* and *cis* interactions are combined, ganglioside-mediated cell–cell recognition can result in changes in cell signaling and cell physiology. One cell–cell recognition system in which gangliosides have been implicated is that between myelin and axons.

Myelin, the multilamellar membrane that wraps many nerve axons in vertebrates, is required for rapid nerve conductance, allowing slender axons to carry electrical signals over long distances [6]. Myelination of axons by Schwann cells (in the peripheral nervous system, PNS) or oligodendrocytes (in the central nervous system, CNS) results in segmental stretches of myelin (internodes) separated by narrow gaps, the nodes of Ranvier (Fig. 1). These gaps are highly structured; they are bordered by loops of myelin that form a seal surrounding the circumference of the underlying axon [6]. Myelination not only insulates axon membranes in internodes, but also regulates the lateral distribution of membrane molecules at nodes of Ranvier. Voltage-gated sodium channels are clustered at the nodes, allowing depolarizing currents to jump from node-to-node, the mechanism for rapid "saltatory" conduction of an action potential across long distances. The loops of myelin that seal the edge of each node define the paranodal region, which is characterized by its own set of molecules and tight membrane-to-membrane adhesion between the axon and myelin. A specialized segment of axon adjacent to the paranode (further from the node), termed the juxtaparanode, is characterized by the presence of voltage-gated potassium channels that help return the membrane to its resting state after depolarization. Together, this complex of membrane molecules supports highly efficient and rapid action potential propagation.

In addition to insulating axons and regulating molecular distributions at nodes of Ranvier, myelin nurtures the axons it ensheathes [7]. When myelin is lost (e.g. by disease), axons suffer. The progressive long-term deficits of "pure demyelinating"

Abbreviations: ARI, axon regeneration inhibitor; MAG, myelin-associated glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; P4, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol

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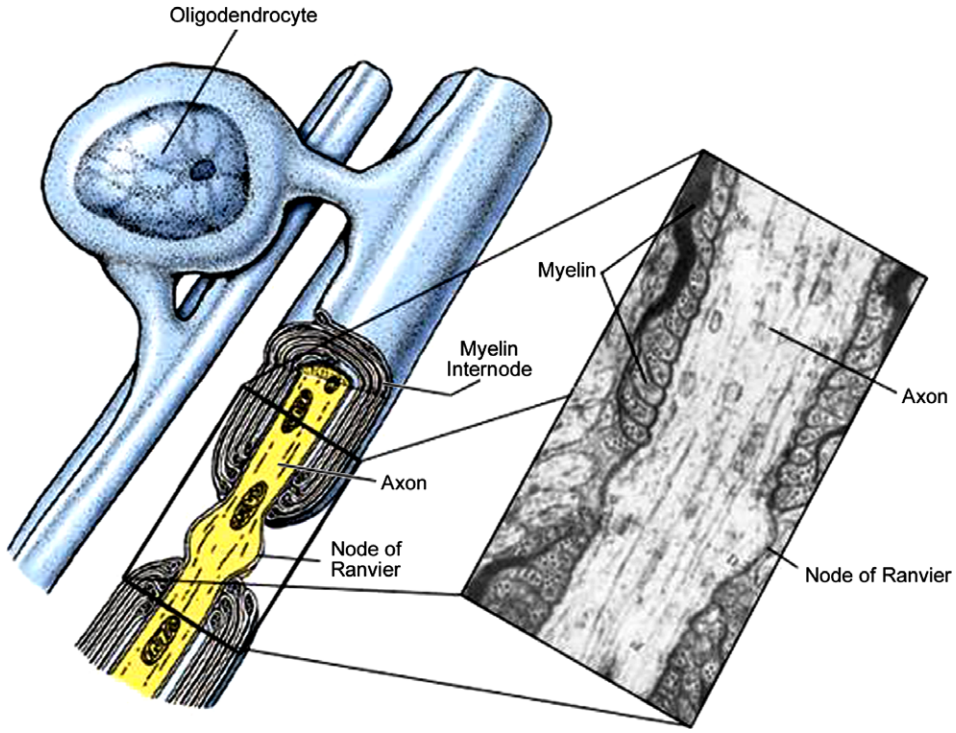

Fig. 1. Myelin and nodes of Ranvier in the CNS. An oligodendrocyte (blue) ensheathing a neuronal axon (yellow) is shown. Axon ensheathment occurs in stretches along the axon (myelin internodes) that are interrupted by specialized gaps, nodes of Ranvier. The ultrastructural insert shows characteristic paranodal myelin loops adhering firmly to an axon at the edge of the node. Reproduced with permission [56].

diseases, such as multiple sclerosis, are believed to be due to the chronic and irreversible secondary loss of axons. Studies of human disease and animal models of disease indicate that myelin acts as a stabilizing factor required for long-term survival of myelinated axons.

Whereas axon stability is required for healthy nervous system function, stabilization signals may be counterproductive after injury. The injured CNS is a highly inhibitory environment for axon regeneration, in part because molecules on residual myelin at the injury site specifically signal axons to halt regrowth [8]. Understanding myelin-mediated "stop" signals and the molecular pathways responsible provides new therapeutic targets to enhance recovery from CNS trauma, such as spinal cord injury [9].

Sets of complementary molecules on apposing axon and myelin surfaces are essential for accurate and efficient myelination, long-term axon stability, and regulation of axon outgrowth. Biochemical, cell biological and genetic data indicate that gangliosides (on the axon surface) and a complementary binding protein, myelin-associated glycoprotein (MAG, on myelin) contribute to these functions [10].

2. Brain gangliosides

Gangliosides are glycosphingolipids that carry one or more sialic acid residue(s) in their oligosaccharide structure [3]. In the brain, ganglioside structures and expression levels are conserved among mammals [1], with four gangliosides – GM1, GD1a, GD1b and GT1b – making up the vast majority (96% of brain gangliosides in man, see Fig. 2 for ganglioside structures). The ceramide lipid moiety of brain gangliosides is most often comprised of an 18- or 20-carbon sphingosine and a saturated fatty acid amide, such as C18:0. The biophysical properties of the ceramide moiety result in ganglioside clustering in the plane of the membrane [3], a topic discussed elsewhere in this Special Issue.

Ganglioside biosynthesis is step-wise [11], starting with transfer of glucose to ceramide (Fig. 2). Subsequent additions of galactose, sialic acid, and N-acetylgalactosamine from their nucleotide sugar donors to the growing saccharide chain generate the penta-, hexa- and heptasaccharide glycans of the major brain gangliosides. Simpler ganglioside structures (GM3 and GD3, see Fig. 2) predominate early in development with the mature major brain gangliosides emerging during the third trimester in rodents [12]. In humans, a sharp increase in brain ganglioside expression begins in the third trimester and extends through the first two postnatal years [13], the same period in which myelination is most active [14].

3. Brain gangliosides are receptors for myelin-associated glycoprotein (MAG)

Vertebrate cell surfaces, including neurons and their axons, are endowed with a rich and diverse glycan coat, the glycocalyx [15]. Cell surface glycan structures often act as receptors for complementary glycan binding proteins, lectins, expressed on apposing cell surfaces. Lectin–glycan binding mediates cell–cell recognition and regulates cell physiology. Several families of lectins, comprising over 100 different proteins with varied glycan binding specificities, are coded in the human genome.

Members of one lectin family, the siglecs, bind with high specificity to glycans terminated with sialic acids [16]. Sialic acids are the most abundant terminal sugar in mammalian glycans, and exist in diverse groupings with other sugars [17]. Siglec family members take advantage of sialoglycan structural diversity; some bind specifically to α2-6-linked sialic acids, and others to α2-3- or α2-8-linked sialic acids. In the siglec family, only one member, myelin-associated glycoprotein (MAG, Siglec-4) is expressed in the brain, and appears to be designed to bind to the major brain gangliosides GD1a and GT1b.

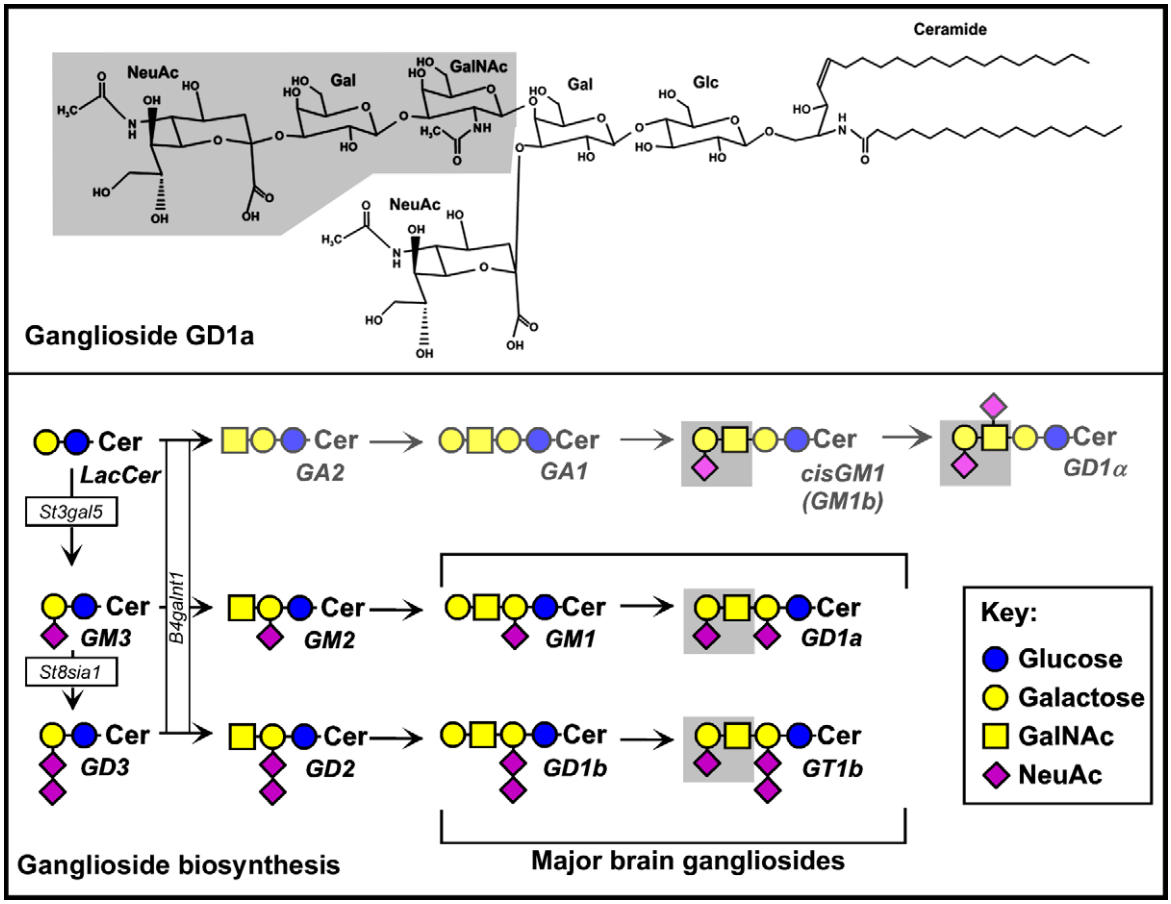

Fig. 2. Ganglioside structures and their biosynthesis. Top: The structure of GD1a is shown with the MAG-binding determinant (NeuAc α2-3 Gal β1-3 GalNAc) shaded. Bottom: Biosynthetic pathways to the major brain gangliosides. The MAG-binding determinant is shaded, and the glycosyltransferases discussed in the text, *B4galnt1*, *St8sia1*, and *St3gal5* are shown. *St3gal5*-null mice lack normal major brain gangliosides, but instead synthesize an equivalent amount of the otherwise rare gangliosides *cis*GM1 (GM1b) and GD1α, both of which carry the MAG-binding determinant. Reproduced with permission [10].

Initial studies revealed that MAG binds specifically to α2–3-linked sialic acid, and has high affinity for glycans that carry the terminal sequence "NeuAc α2-3 Gal β1-3 GalNAc" [18]. On nerve cells and axons this terminus is most abundantly expressed on gangliosides GD1a and GT1b [19]. In vitro, MAG binds avidly to GD1a and GT1b, but not to closely related gangliosides (e.g. GM1 and GD1b), which lack the "NeuAc α2-3 Gal β1-3 GalNAc" terminus [20,21]. Altering the hydroxyls, the N-acyl group or the carboxylic acid on the terminal sialic acid of a ganglioside eliminates MAG binding, indicating that MAG is highly evolved to engage sialoglycans with high specificity [22]. The binding data led to the hypothesis that MAG, on myelin, functions *in vivo* by binding to gangliosides GD1a and/or GT1b expressed on the axon surface [20]. Genetic studies are consistent with this hypothesis.

4. Genetic studies implicate gangliosides in axon–myelin interactions

Gangliosides are biosynthesized step-wise by a series of specific glycosyltransferases (Fig. 2). The functions of gangliosides can be inferred by studying the phenotypes of mice engineered to lack one or more of these enzymes [23,24]. A particularly revealing mutant lacks expression of the N-acetylgalactosaminyltransferase required to initiate the "NeuAc α2-3 Gal β1-3 GalNAc" terminus on gangliosides [25–27]. When the gene responsible, *B4galnt1* (previously called *Galgt1* or GM2/GD2 synthase) is disrupted, none of the major brain gangliosides are expressed. The total brain ganglioside concentration remains the same as in wild-type mice, but with the simple gangliosides GM3 and GD3 accumulating behind the biosynthetic block [28]. Since GD3 does not support MAG binding, and GM3 supports relatively poor MAG binding [21], the expectation was that *B4galnt1*-null mice would have impaired axon–myelin interactions. This is what was found [27]. Although *B4galnt1*-null mice are born in Mendelian frequencies and survive to adulthood, they display progressive neuropathy characteristic of a loss of axon–myelin stability. By the time they are one year of age, they have significantly impaired hindlimb mobility. A comparison of *B4galnt1*-null, *MAG*-null and *B4galnt1*/*MAG* double null mice reveals striking similarities in their phenotypes [29]. They all display significant axon degeneration in the CNS and PNS, reduced axon caliber, reduced axon neurofilament spacing, and motor behavioral deficits consistent with neuropathy, such as impaired motor coordination and poor hindlimb reflexes. The phenotypic similarity between *B4galnt1*-null, *MAG*-null and double null mice is consistent with the hypothesis that MAG and gangliosides participate in the same pathway, enhancing axon–myelin stability. The intracellular signaling pathways downstream of MAG-ganglioside binding that lead to enhanced stability have not been established.

In addition to supporting long-term axon stability, complex gangliosides contribute to appropriate axon–myelin interactions at nodes of Ranvier [30]. Whereas wild-type nodes are characterized by well-organized paranodal loops with characteristic "transverse bands" mediating axon–myelin attachments, *B4galnt1*-null mice often display poorly structured paranodal loops that fail to attach to the axon or that attach but lack transverse bands. Immunohistochemical localization of sodium and potas-

sium channels supports the conclusion that complex gangliosides regulate nodal structure. In *B4galnt1*-null mice, sodium channels, normally restricted to narrow band at the node, are broadened. Potassium channels, normally spaced away from the node by the paranode, also are broadened to populate the paranode. It is likely that these structural and molecular deficits at nodes of Ranvier underlie the reduced motor nerve conduction velocity measured in electrophysiological studies of *B4galnt1*-null mice [25,30].

The phenotype of *B4galnt1*-null mice revealed that complex gangliosides are required for axon–myelin long-term stability. The data are consistent with the hypothesis that the observed neuronal deficits are due to the loss of MAG interaction with its axonal receptors, GD1a and GT1b. However, loss of the *B4galnt1* gene product, GM2/GD2 synthase, results in loss of all complex gangliosides, not just those that interact with MAG [25,26,28]. To infer which gangliosides are responsible for axon–myelin stability, it is instructive to consider mice with mutations in genes coding other enzymes in the ganglioside biosynthetic pathway (see Fig. 2) [23,24].

B4galnt1-null mice express about the same total brain ganglioside concentration as wild-type mice, building up the simpler species behind the metabolic block [28]. Likewise, except for mice with no brain gangliosides (see below), mice with disruptions in other ganglioside biosynthetic genes express about the same total concentration of brain gangliosides, building up gangliosides behind each genetic block [31]. Thus, *St8sia1*-null (GD3 synthase-deficient) mice build up GM1 and GD1a in the absence of "b-series" gangliosides. These mice express ample amounts of the MAG-binding ganglioside GD1a, and do not have overt axon–myelin deficits. *St3gal5*-null (GM3 synthase-deficient) mice, which might be expected to lack all brain gangliosides, instead express the same total brain ganglioside concentration as wild-type mice, but in the form of the normally rare gangliosides *cis*GM1 (GM1b) and GD1α (see pathways, Fig. 2) [32]. These mice do not have overt axon–myelin deficits. Notably, GD1α, which is amply expressed in *St3gal5*-null mice [32], supports avid MAG-binding. To block all "ganglio-series" brain ganglioside biosynthesis, Yamashita et al. engineered *St3gal5*/*B4galnt1* double null mice (see Fig. 2) [33]. These mice fail to express significant amounts of brain gangliosides and demonstrate early axonal degeneration and severely perturbed axon–myelin interactions. These studies, taken together, are consistent with a role for MAG-binding gangliosides (GD1a and GT1b) in axon–myelin stability.

Human genetic deficits in the enzymes that biosynthesize major brain gangliosides are exceedingly rare. Nevertheless, a small clade of individuals was identified with a disrupted *St3gal5* gene (see Fig. 2) [34]. The affected children all displayed seizures before they were one year old. This was followed by diffuse brain atrophy, areflexia, and developmental regression. Seizure activity has been observed in ganglioside-deficient mice as well [31]. Although additional data will be needed to correlate ganglioside structure to ganglioside function in this rare human disorder, a critical role of gangliosides in human brain function has been established.

5. Gangliosides as receptors for MAG inhibition of axon outgrowth

The injured adult vertebrate central nervous system is an inhibitory environment for axon regeneration, resulting in poor recovery after, for example, spinal cord injury [8,35]. In part, this is due to specific "axon regeneration inhibitors" (ARIs) that accumulate at injury sites, including the protein molecules MAG, Nogo, and oligodendrocyte-myelin glycoprotein (OMgp) on residual myelin (Fig. 3) and chondroitin sulfate proteoglycans on reactive astrocytes. In each case, ARIs bind to receptors on axons, signaling them to halt regeneration. Since MAG binds with high specificity to the major axon gangliosides GD1a and GT1b, we proposed that MAG engagement of these gangliosides is responsible for inhibiting axon regeneration [20]. Several lines of data support this hypothesis [9,36–38]. Although inhibition is multifaceted and complex, MAG-ganglioside binding appears to be one pathway leading to axon outgrowth inhibition.

Evidence for ganglioside involvement in axon outgrowth inhibition came from in vitro experiments using primary nerve cells isolated from newborn rats and mice. Binding of an molecularly expressed form of MAG (MAG-Fc) to some types of nerve cells was completely reversed by pretreatment of the nerve cells with sialidase [18,39]. In functional studies, inhibition of axon outgrowth induced by MAG was reversed by sialidase, by the ganglioside biosynthetic inhibitor P4 (1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol), and by antibodies to GD1a or GT1b [36,37]. Furthermore, nerve cells isolated from *B4galnt1*-null mice, which lack complex gangliosides including GD1a and GT1b, were less responsive to MAG-mediated inhibition [37]. Together, these data implicate gangliosides as functional MAG receptors responsible for axon outgrowth inhibition. It has been proposed that MAG engages and clusters GD1a or GT1b at the axon surface, halting axon outgrowth. In support of this concept, antibody-mediated crosslinking of gangliosides GD1a or GT1b at the nerve cell surface mimicked MAG inhibition, and the same ultimate downstream intracellular signaling pathway, activation of the small GTPase RhoA, was found to be responsible for axon outgrowth inhibition by both MAG and anti-ganglioside antibodies [36,37,40,41].

Although gangliosides are strongly implicated as MAG receptors, and can mediate axon outgrowth inhibition, there are other MAG

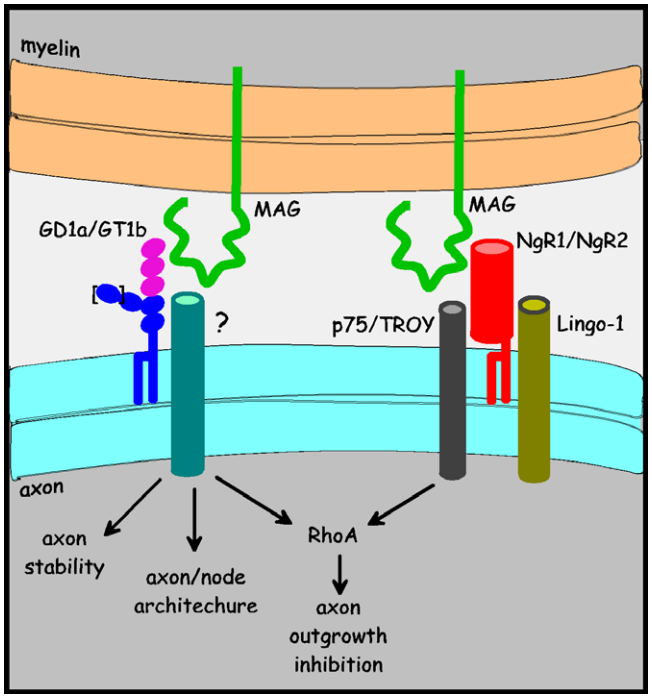

Fig. 3. Dual MAG receptor model. MAG is envisioned as interacting independently with gangliosides (GD1a or GT1b, MAG-binding determinant highlighted) and with NgR's (NgR1 or NgR2). In each pathway, MAG engagement results in transmembrane signals. For example, NgR1 interacts with transmembrane signaling molecules $p75^{NTR}$ and Lingo-1 to transduce a signal that results in RhoA activation and inhibition of axon outgrowth. Gangliosides may signal via some of the same components, or via yet undefined components as shown, to activate RhoA, modulate axon and node of Ranvier structures, and stabilize axons. Additional modulating factors (cAMP, calcium, PKC) and potential pathway crosstalk are not shown (see [8]). Reproduced with permission [10].

receptors [8,42], and not all nerve cells use the same MAG receptor [40,43]. Whereas MAG inhibition of axon outgrowth from cerebellar granule and hippocampal neurons is largely via gangliosides, as evidenced by reversal of inhibition by sialidase or P4, MAG inhibition of retinal and dorsal root ganglion axon outgrowth is largely resistant to these treatments. Neurons that did not depend on gangliosides to respond to MAG, appeared to respond via a second class of MAG receptors, the glycophosphatidylinositol (GPI) linked NgR family of proteins. Some data indicate that ganglioside-mediated and NgR-mediated inhibition of axon regeneration are independent [40]. Other data suggest that NgR and gangliosides, both of which partition in membrane rafts [44], cooperate to signal axon outgrowth inhibition [41]. Recently, additional MAG receptors were reported [42,45]. Sorting out which receptors regulate which nerve cell responses in different circumstances will be important in considering potential therapeutic implications of gangliosides as regulators of axon regeneration after injury.

6. Modulating gangliosides to enhance axon outgrowth after injury

If gangliosides act as functional MAG receptors mediating inhibition of axon outgrowth after nervous system injury, would blocking or modifying gangliosides enhance recovery? Evidence from in vitro studies suggests that recovery in response to ganglioside modification would depend on which nerve cell axons were injured [40,43]. Furthermore, given the number and complexity of axon outgrowth inhibitory molecules and pathways (Fig. 3), ganglioside modification might have to be combined with other anti-ARI therapies to be effective [8,9]. Despite these caveats, evidence in support of ganglioside modification therapy comes from a study in which sialidase, one of the most robust in vitro treatments to reverse MAG-mediated axon outgrowth inhibition, enhances motor axon outgrowth when delivered to a nerve injury in vivo [46].

Brachial plexus avulsion is an injury in which the nerves leading to and from the arm are yanked out of the spinal cord, most commonly during a motor vehicle accident, leaving the arm completely paralyzed [47]. To model this injury in rats, nerve roots at the shoulder level were exposed and cut flush where they entered/exited the spinal cord [46]. A nerve graft was inserted into the spinal cord at the injury site in an attempt to coax motoneuron axons out of the spinal cord. Sialidase was injected onto the insertion site immediately after graft insertion, and then pumped onto the site continually for 14 days thereafter. After 4 weeks, neurons that extended new axons from the spinal cord into the graft were labeled by retrograde fluorescent labeling and counted. The results were striking. Without sialidase ~170 axons exited the spinal cord into the graft, whereas with sialidase treatment >400 spinal axons entered the graft. The morphology of the labeled cells indicated that the axons were from motor neurons. The number of axons exiting the spinal cord and entering the graft in sialidase-treated animals was significant, with recovery of perhaps half of the number of motor axons that exit the spinal cord at that level in an uninjured rat.

Since sialidase cleaves sialic acid from sialoglycoproteins as well as gangliosides (sialoglycolipids), it is not possible to ascribe the enhanced spinal axon outgrowth to gangliosides per se. Furthermore, sialidase treatment cleaves only the external sialic acids from complex gangliosides, leaving an increased concentration of the ganglioside GM1, which itself may affect axon outgrowth [48–50]. Nevertheless, since GD1a and GT1b are major sialoglycans of nerve tissue, and have been implicated in axon outgrowth control via their interaction with MAG, the data are consistent with a role for these gangliosides in inhibiting axon outgrowth after injury in vivo, and identify these gangliosides as potential therapeutic targets.

If specific gangliosides are required for proper axon–myelin architecture and long-term axon–myelin stability, how can their destruction enhance recovery from injury? It is hoped that ganglioside-modifying treatments such as sialidase might be administered during a critical period after injury to enhance plasticity and encourage axon outgrowth. Thereafter, treatment would be halted to allow expression of the normal assortment of major brain gangliosides required for long-term axon–myelin health.

7. Challenges and perspectives

Biochemical, cellular and genetic data (see above) confirm that complex brain gangliosides play important roles in the long-term stability of axons and in the regulation of axon regeneration. We infer that engaging GD1a or GT1b on the outer leaflet of the plasma membrane generates transmembrane signals that result in fundamental changes in the cytoskeleton that halt axon outgrowth and support axon survival and function. Since gangliosides do not span the membrane, biochemical mechanisms must exist to detect ganglioside engagement and convert binding to transmembrane signals [4]. This may occur by altering direct lateral associations of gangliosides with transmembrane signaling molecules. Alternatively, gangliosides constitutively associate with each other and certain other outer leaflet molecules to form microdomains, also called lipid rafts. These rafts accumulate signaling molecules on the inner leaflet, such as Src-family kinases. Aggregating gangliosides on the outer membrane leaflet may alter signaling molecule associations on the inner leaflet. There are precedents for both direct and raft-mediated ganglioside signaling.

Gangliosides can associate directly with transmembrane signaling molecules and modulate their downstream signaling. For example, insulin and EGF receptors associate laterally with ganglioside GM3, resulting in decreased tyrosine kinase activity in response to insulin and EGF, respectively [32,51,52]. In contrast, the activity of the TrkA neurotrophin receptor, which is responsible for NGF-induced axon outgrowth, is enhanced by its association with ganglioside GM1 [48].

More directly relevant to axon–myelin interactions, there is evidence for the physical association of gangliosides with $p75^{NTR}$ [53] and NgR1 [41], two transmembrane signaling molecules on axons implied in axon outgrowth inhibition by MAG in some cell types. However, ganglioside-mediated axon outgrowth inhibition (at least in some neurons) is clearly independent of $p75^{NTR}$ and NgR [40,43]. This does not exclude the potential role of ganglioside–$p75^{NTR}$ or ganglioside–NgR associations in axon–myelin interactions, but other mechanisms must also exist.

Engaging gangliosides on the outer leaflet of the plasma membrane can regulate the activity of lipid-raft-associated signaling molecules on the inner leaflet. For example, antibody-induced crosslinking of GM3 on melanoma cells activates Src and RhoA [54], and crosslinking GD3 on cerebellar neurons activates Lyn [55]. In a ganglioside-mediated axon outgrowth study, the extracellular matrix molecule laminin-1 was found to bind and cluster GM1, resulting in recruitment of TrkA and β1 integrin to rafts and activation of Lyn on the inner leaflet [50]. These data provide a precedent for raft-mediated outside-in signaling initiated by ganglioside clustering.

The signaling molecules and mechanisms that are responsible for converting MAG-ganglioside binding to enhanced axon–myelin stability and axon outgrowth inhibition have not been fully described. Given the above precedents, it is likely that clustering gangliosides on the axon surface impacts transmembrane receptors, lipid raft signaling molecules, or both. Identifying the specific molecules and mechanisms involved may provide additional

opportunities to enhance long-term axon stability and improve axon regeneration after injury.

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